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Endothelin-1-(1–31), a novel vasoactive peptide, increases $[Ca^{2+}]_i$ in human coronary artery smooth muscle cells

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Abstract

We have previously found that human chymase cleaves big endothelins at the Tyr^{31}–Gly^{32} bond and produces 31-amino acid long endothelins-(1–31), without any further degradation products. In this study, we investigated the effect of synthetic endothelin-1-(1–31) on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured human coronary artery smooth muscle cells. Endothelin-1-(1–31) increased $[Ca^{2+}]_i$ in a concentration-dependent manner (10^{-14} to 10^{-10} M). This endothelin-1-(1–31)-induced $[Ca^{2+}]_i$ increase was not affected by phosphoramidon (*N*-(α-Rhamnopyranosyloxyhydroxyphosphinyl)-L-Leucyl-L-Tryptophan), an inhibitor of endothelin-converting enzyme. It was, however, inhibited by 10^{-10} M BQ123 (Cyclo-(-D-Trp-D-Asp(ONa)-Pro-D-Val-Leu-)), an endothelin ET_A receptor antagonist, but not by 10^{-10} M BQ788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γMeLeu-D-Trp(COOMe)-D-Nle-ONa), an endothelin ET_B receptor antagonist. These results suggest that endothelin-1-(1–31) by itself exhibits vasoactive properties probably through endothelin ET_A receptors. Since human chymase has been reported to play a role in atherosclerosis, endothelin-1-(1–31) may be one of the candidate substances for its cause. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Endothelin-1; Endothelin-1-(1–31); Chymase, human; Microscopy, confocal; Coronary artery smooth muscle cell, human

1. Introduction

Endothelin-1 is a 21-amino acid polypeptide which exhibits various physiological actions, such as vascular contraction (Yanagisawa et al., 1988), cardiac hypertrophy (Arai et al., 1995) and mitogenesis (Chua et al., 1992). Human endothelin-1 is generated from the 38-amino acid precursor, big endothelin-1, through cleavage of the Trp^{21}–Val^{22} bond via the action of endothelin-converting enzyme. Although endothelin-converting enzyme was originally shown to be a membrane-bound metalloprotease (Yanagisawa et al., 1988), several other metalloproteases have also been postulated to catalyze the formation of endothelin-1 from big endothelin-1 (Matsumura et al., 1990). Rat mast cell chymase has also been reported as a putative converter of big endothelin-1 to endothelin-1 (Wypij et al., 1992).

We have recently reported that human mast cell chymase, unlike rat mast cell chymases, selectively cleaves big endothelins at the Tyr^{31}–Gly^{32} bond to produce novel trachea-constricting 31-amino acid long endothelins, endothelins-(1–31), without any further degradation products (Nakano et al., 1997). Furthermore, endothelins-(1–31) have been isolated from various human organs, such as lung and heart (Okishima et al., manuscript in preparation). Since endothelin-1 has been shown to play a significant role in the paracrine regulation of cardiovascular functions in humans (Uchida et al., 1988), endothelin-1-(1–31) may possess biological activities in human tissues. In the present study, we examined the effect of synthetic endothelin-1-(1–31) on the intracellular free calcium concentration ($[Ca^{2+}]_i$) in cultured human coronary artery smooth muscle cells, using confocal laser microscopy.

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2. Materials and methods

2.1. Cell culture and loading of the Ca^{2+} indicator fluo-3 into cells

Human coronary artery smooth muscle cells at passage 4 were obtained as a commercially available product from Clonetics (San Diego, CA, USA). Cells were plated in 25-cm^2 tissue culture flasks at a density of 5×10^3 cells/cm^2 in MCDB131 medium supplemented with 5% heat-inactivated fetal calf serum, 0.5 ng/ml epidermal growth factor, 1 ng/ml basic fibroblast growth factor, 5 μg/ml insulin, 50 μg/ml gentamicin and 0.25 μg/ml amphotericin B. The cells were incubated at 37°C in 5% CO_2 and the medium was replaced every other day until the cells were 60–80% confluent. The cells were then removed from the flasks with 0.025% trypsin plus 0.01% EDTA and seeded onto glass cover-slips attached to 35-mm tissue culture dishes coated with poly-L-lysine. All experiments were performed with the cells in passage 5–15 and at 2–3 days post-confluency. For dye loading, the culture medium was removed from the dishes and replaced with modified Krebs–Henseleit bicarbonate buffer solution (K–H solution) (135 mM NaCl, 5.6 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.2 mM $CaCl_2$, 10 mM glucose, adjusted with HCl to pH 7.40) oxygenated with 95% O_2 and 5% CO_2 gas mixture. The cells were then loaded with 4 μM of fluo-3/acetoxymethylester at 37°C. After loading for 30 min, the solution was exchanged for a dye-free K–H solution, and the cells were allowed to de-esterify the indicator for an additional 10 min.

2.2. Measurement of fluorescence intensity with confocal laser microscopy

Fluorescence intensity was analyzed with confocal laser microscopy as described previously (Tanaka et al., 1997). Briefly, a confocal imaging system (Nikon RCM 8000, Tokyo, Japan) with an Argon-ion laser was attached to an inverted microscope (Nikon TMD300, Diaphot). A culture dish of cells in 1 ml of K–H solution was placed on the stage of the microscope and the cells were excited at 488 nm by the laser. Emission at wavelengths longer than 520 nm was then detected by a photomultiplier. After measurement of stable baseline fluorescence intensity, 10 μl of an agent was added to the extracellular medium, and the fluorescence intensity was recorded. After 1 min, the same cells were stimulated by addition of 10 μM of ionomycin and the relative fluorescence intensity was calculated. Calibration of the fluo-3 fluorescence intensity, to estimate $[Ca^{2+}]_i$, was calculated from the difference between F_{max} and F_{min}. To estimate F_{min}, the cells were washed five times with Ca^{2+}-free K–H solution containing 3 mM EGTA and the resting $[Ca^{2+}]_i$ was determined 2 min later. F_{max} was estimated from the intensity at 1 min after addition of ionomycin in each experiment. Results are expressed as a percentage of the difference between F_{max} and F_{min}.

2.3. Chemicals

Human endothelin-1, big endothelin-1 and phosphoramidon (*N*-(α-Rhamnopyranosyloxyhydroxyphosphinyl)-L-Leucyl-L-Tryptophan) were obtained from Peptide Institute (Osaka, Japan). Endothelin-1-(1–31) was synthesized by solid-phase procedures at Peptide Institute. Fluo-3/acetoxymethylester was purchased from Wako Pure Chemical (Osaka, Japan). BQ123 (Cyclo-(D-Trp-D-Asp(ONa)-Pro-D-Val-Leu-)) and BQ788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γMeLeu-D-Trp(COOMe)-D-Nle-ONa) were gifts from Banyu Pharmaceutical (Tsukuba, Japan). All other chemicals used were commercial products of reagent grade.

2.4. Statistics

Data are presented as means ± S.E. for a total of 25 cells observed in five separate experiments. One-way analysis of variance was used to determine significance among groups, after which the modified *t*-test with the Bonferroni correction was used for comparison between individual groups. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

Fig. 1 shows the effect of endothelin-1-(1–31) on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) compared with that of endothelin-1 and big endothelin-1. Endothelin-1-(1–31) at concentrations between 10^{-14} and 10^{-10} M

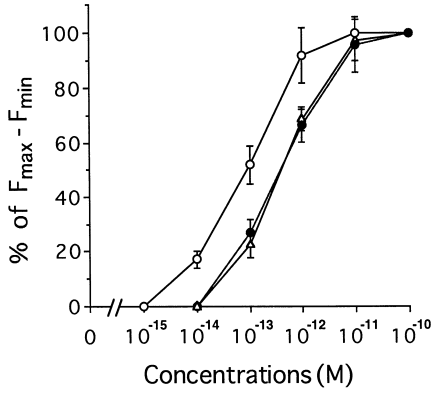

Fig. 1. Concentration–response curves for increases in $[Ca^{2+}]_i$ induced by endothelin-1-(1–31)(●), endothelin-1(○) and big endothelin-1(△) in cultured human coronary artery smooth muscle cells. Values are expressed as % difference of F_{max} and F_{min} as described in the text (means ± S.E. of a total of 25 cells in five separate experiments). F_{max} was obtained from the fluorescence intensity after addition of 10 μM of ionomycin to the incubation medium.

caused an increase in $[Ca^{2+}]_i$ in a concentration-dependent manner. The $[Ca^{2+}]_i$ increase induced by endothelin-1-(1–31) reached a peak within 10 s and then gradually decreased to the baseline resting value within 1 min. In addition, the endothelin-1-(1–31)-induced $[Ca^{2+}]_i$ increase was not affected by removal of Ca^{2+} from the medium (data not shown). Endothelin-1, however, was about 10-times more potent than endothelin-1-(1–31) in the concentrations used. The concentration–response curve of $[Ca^{2+}]_i$ for big endothelin-1 was similar to that for endothelin-1-(1–31).

To investigate the possibility that the $[Ca^{2+}]_i$ increase induced by endothelin-1-(1–31) may be due to further degradation of endothelin-1-(1–31) to endothelin-1 by endothelin-converting enzyme in the medium or in the cells, we examined the effect of an inhibitor of endothelin-converting enzyme, phosphoramidon (Matsumura et al., 1991), on the endothelin-1-(1–31)-induced increase in $[Ca^{2+}]_i$. As shown in Fig. 2, phosphoramidon at 10^{-5} M failed to inhibit the increase in $[Ca^{2+}]_i$ induced by endothelin-1-(1–31) at a concentration of 10^{-12} M.

To determine whether the effect of endothelin-1-(1–31) is a receptor-mediated phenomenon, we examined the effects of endothelin receptor antagonists on the increase in $[Ca^{2+}]_i$ evoked by endothelin-1-(1–31). Since it has been reported that there are at least two main subtypes of endothelin receptors, termed endothelin ET_A and ET_B receptors (Watanabe et al., 1989), we examined the effects of a specific endothelin ET_A receptor antagonist, BQ123 (Ihara et al., 1992), and a specific endothelin ET_B receptor antagonist, BQ788 (Ishikawa et al., 1994), on the endothelin-1-(1–31)-induced increase in $[Ca^{2+}]_i$. As shown in Fig. 2, BQ123 at 10^{-10} M almost abolished the increase in $[Ca^{2+}]_i$ induced by 10^{-12} M of endothelin-1-(1–31), whereas BQ788 at the same concentration had no effect on the increase in $[Ca^{2+}]_i$.

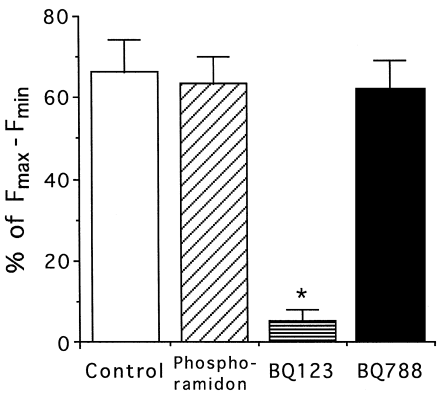

Fig. 2. Effects of phosphoramidon (10^{-5} M), BQ123 (10^{-10} M) and BQ788 (10^{-10} M) on endothelin-1-(1–31)-induced increases in $[Ca^{2+}]_i$ in cultured human coronary artery smooth muscle cells. Values are expressed as % difference of F_{max} and F_{min} as described in the text (means ± S.E. of a total of 25 cells in five separate experiments). The control value is the fluorescence intensity obtained with a half-maximal dose (10^{-12} M) of endothelin-1-(1–31). Each drug was added to the incubation medium throughout dye loading and in each experiment. (* $P < 0.05$ from the control value).

4. Discussion

Human chymase is highly efficient in converting angiotensin I to angiotensin II (Urata et al., 1990b), and the chymase-dependent angiotensin II-forming pathway is recognized as a major pathway for angiotensin II formation in cardiovascular tissues (Urata et al., 1990a). We have previously found that human mast cell chymase also specifically converts big endothelins to novel trachea-constricting 31-amino acid long peptides, endothelins-(1–31), which are longer than the well-known 21-amino acid endothelins (Nakano et al., 1997). It has also been reported that a serine protease in human lungs hydrolyzes big endothelin-1 to a fragment of endothelin-1-(1–31) which has contractile activity in pulmonary artery (Hanson et al., 1997). Since endothelin-1 and angiotensin II possess a wide variety of biological activities, including cardiac hypertrophy (Arai et al., 1995), and have a role in the pathogenesis of atherosclerosis (Weissberg et al., 1990), endothelins-(1–31) may also be novel vasoactive peptides in the endothelin family. Since the increase in $[Ca^{2+}]_i$ caused by endothelin-1 has been implicated in vascular contraction (Simonson and Dunn, 1990) and vascular smooth muscle cell proliferation (Komuro et al., 1989), we investigated the effect of synthetic endothelin-1-(1–31) on $[Ca^{2+}]_i$ in cultured human coronary artery cells, using confocal laser microscopy.

As shown in Fig. 1, the results revealed that the activity of endothelin-1-(1–31) to increase $[Ca^{2+}]_i$ was almost 10-times less potent than that of endothelin-1 and similar to that of big endothelin-1. However, the potency of endothelin-1-(1–31) to raise $[Ca^{2+}]_i$ was 100-times and 10 000-times more pronounced in the concentrations used than those of angiotensin II and noradrenaline, respectively (data not shown). Although earlier studies used relatively high concentrations (nanomolar range) of endothelin-1 (Gardner et al., 1992; Xuan et al., 1994), our results revealed that endothelin-1-(1–31), as well as endothelin-1, increased $[Ca^{2+}]_i$ in the picomolar range. These results are consistent with other observations that endothelin-1 causes contraction of rat renal afferent arterioles at concentrations around 10^{-12} M (Lanese et al., 1992). We also confirmed that endothelin-1 contracted rabbit afferent arterioles at concentrations higher than 10^{-13} M (unpublished data). Considering the findings that tissue or blood concentrations of endothelin-1 are in the picomolar range (Holm and Franco-Cereceda, 1996), it is reasonable to speculate that the physiological effect of endothelin-1-(1–31) may develop at picomolar concentrations.

It is important to elucidate whether the effect of endothelin-1-(1–31) is a result of its extracellular conversion to endothelin-1, or whether endothelin-1-(1–31) itself acts directly on cells. As shown in Fig. 2, phosphoramidon, an

inhibitor of metalloendopeptidases, and endothelin-converting enzyme (Matsumura et al., 1991), at a concentration of 10^{-5} M had almost no effect on the increase in $[Ca^{2+}]_i$ elicited by endothelin-1-(1–31), although phosphoramidon at the same concentration effectively inhibits the contractile activity of big endothelin-1 (Advenier et al., 1992; Nakano et al., 1997). The results are consistent with the findings in the previous report that endothelin-converting enzyme requires the C-terminal structure of big endothelin-1 for enzyme recognition and is not able to cleave endothelin-1-(1–31) (Xu et al., 1994). Taken together, the results indicate that the activity of endothelin-1-(1–31) to increase $[Ca^{2+}]_i$ is not a consequence of its conversion to endothelin-1 by endothelin-converting enzyme or metalloendopeptidase(s). It should be noted that endothelin-1-(1–31) itself has biological activity in cultured human coronary artery smooth muscle cells, regardless of whether or not it is degraded to endothelin-1.

The above findings are consistent with the hypothesis that endothelin-1-(1–31) binds to its receptor(s) without further proteolytic degradation and induces an increase in $[Ca^{2+}]_i$ in cultured coronary artery smooth muscle cells. As shown in Fig. 2, the $[Ca^{2+}]_i$-increasing effect of endothelin-1-(1–31) was inhibited by 10^{-10} M of BQ123, but not by 10^{-10} M of BQ788, known inhibitors of endothelin ET_A and ET_B receptors, respectively. Although we have no evidence that the receptor of endothelin-1-(1–31) is identical to that of endothelin-1, the results suggest that the cell response induced by endothelin-1-(1–31) is mediated through endothelin ET_A or ET_A-like receptors. Further studies are needed to clarify what type of receptors are involved in the endothelin-1-(1–31)-induced phenomenon.

In conclusion, endothelin-1-(1–31) is a novel putative vasoactive peptide of the endothelin family that may be deeply involved in chymase-related pathophysiological processes in humans. Since chymase plays a significant role in foam cell formation in human coronary atheromas (Kovanen et al., 1995), the pathogenesis of atherosclerosis in coronary artery may be attributable to the effect of bioactive endothelin derivatives, including endothelin-1-(1–31), an endogenous product of mast cell chymase in humans.

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